Dogra A, et al., Adv Ind Biotechnol 2018, 1: 002
DOI: 10.24966/AIB-5665/100002

HSOA
Advances in Industrial Biotechnology

Research Article

A Comparison of Physi-co-Chemical Properties of Free and Chitosan Bead-Immobi-lized Pectin Lyase from *Bacil-lus Cereus*

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Abstract

In this study, high binding efficiency for immobilization of pectin lyase was obtained using chitosan beads. Formaldehyde at a concentration of 10% (v/v) was used as a cross-linking agent. Further, characterization of free and immobilized pectin lyase was carried out to compare their physico-chemical properties. The optimal conditions for activity of free pectin lyase were found to be: glycine-NaOH buffer (50 mM), pH 10.0, incubation time 15 min and reaction temperature 40˚C and for immobilized enzyme: glycine-NaOH buffer (50 mM), pH 10.0, incubation time 15 min and reaction temperature of 50˚C. Pectin lyase showed maximum enzyme activity in the presence of Mg^{2+} ions for both free and immobilized enzyme. Chitosan bead-bound pectin lyase showed 83% binding efficiency. Immobilized pectin lyase retained almost 50% of its original activity up to 4^{th} cycle. The obtained bio-conjugate showed increased optimum temperature and improved thermostability. These properties support the potential application of the immobilized pectin lyase in the pulp industries.

Keywords: Chitosan beads; Degradation; Immobilization; Pectin; Pectin lyase

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Citation: Dogra A, Kohli P, Bhardwaj KK, Gupta R (2018) A Comparison of Physico-Chemical Properties of Free and Chitosan Bead-Immobilized Pectin Lyase from *Bacillus Cereus*. Adv Ind Biotechnol 1: 002.

Received: December 28, 2017; **Accepted:** April 11, 2018; **Published:** April 25, 2018

Introduction

As important biocatalysts, the use of enzymes is widespread in many applications, such as biosensors [1], pharmaceuticals, chemicals and foods [2,3]. Pectinases are considered one of the newly discovered enzymes of fruits and textile industries which are produced by bacteria, pathogenic fungi as well as by plants. Pectinase hydrolyses complex plant pectin polysaccharides into smaller molecules like galacturonic acids [4]. Pectin is structurally and functionally the most complex polysaccharide in plant cell wall [5]. The primary chain of pectin is composed of α-1, 4-linked residues of D-galacturonic acid [6].

Pectinase enzymes are classified into Polygalacturonase (PG), Pectinesterase (PE) and Pectin Lyase (PL) based on their mode of action on the substrate [6]. Pectin lyase (E.C 4.2.2.10) is a pectinolytic enzyme that catalyzes the cleavage of pectin, preferentially highly esterified pectin, producing unsaturated methyl oligogalacturonates through transelimination of glycosidic linkages [6].

Immobilization of enzymes in different matrices is an important method to enhance the stability, selectivity and in some cases the reactivity of enzymes. Pectin lyases have been immobilized by encapsulating in different organic and inorganic polymeric supports, as agar-agar, polyvinyl alcohol foam, sodium alginate and chitosan [7,8]. Out of various materials used for immobilization, chitosan is worthy for its low cost and bio-compatibility. The chitosan is considered as an excellent and inexpensive support for the immobilization of enzymes due to its inert, hydrophilic and biocompatible properties [9]. The immobilization can be carried out by entrapment within chitosan beads or through covalent binding onto the chitosan beads using cross linking agents [10]. In current study, chitosan beads have been used for the immobilization of pectin lyase through covalent binding using formaldehyde as a cross linking agent. Immobilized pectin lyase has broad applications in pulp industries and biotechnology. Pulps prepared with pectinolytic enzymes produce bulkier paper with high opacity and better printability than pulps prepared by an alkaline chemical process. Generally, they show better thermal and pH stabilities and are easier to separate, can be reused and their effect appears to be more suitable for pulp industry.

Materials and Methods

Materials

Pectin lyase producing bacterium *Bacillus cereus* was isolated from the soil sample collected from vicinity of roots of the tree *Grewia optiva* at Distt. Kullu, Himachal Pradesh. Chemicals used in the present investigation were either obtained from Sigma Aldrich (USA) or Himedia (Mumbai, India) and were of high quality and analytical grade.

Purification

The pectin lyase from *Bacillus cereus* was partially purified by ammonium sulphate precipitation method and dialysis. The dialyzed enzyme was further used for immobilization.

Enzyme immobilization

The immobilization of partially purified pectin lyase was carried out by surface adsorption method on different matrices such as agar-agar, celite-545 and chitosan. Chitosan was selected further for immobilization on the basis of binding efficiency. For the preparation of chitosan beads, 1.0 g chitosan was dissolved in 4% of acetic acid solution and kept overnight. The mixture was added drop wise into 10% chilled NaOH solution while stirring continuously and was kept for 30 min. Beads were filtered and then washed twice with distilled water. The beads were cross linked with 10% (v/v) formaldehyde solution and incubated at 30˚C temperature for 5 hr. The resulting cross linked sample was washed with buffer. Finally, the beads were transferred into 5 ml of enzyme and then kept for immobilization for 20 hr incubation at 30˚C. The beads were washed with 50 mM glycine-NaOH buffer and stored in the same buffer.

Enzyme assay

Pectin lyase assay was performed by spectrophotometric method using pectin as substrate and TBA (thiobarbituric acid) as colouring agent at 550 nm [11]. The concentration of protein was estimated by dye binding method using standard Bovine Serum Albumin (BSA) [12].

One Unit (U) of enzyme activity is defined as µmol (s) of galacturonic acid released per minute by 1 ml of soluble enzyme or 1 mg of immobilized enzyme (weight of matrix included) under standard assay conditions.

Binding efficiency

The binding efficiency was calculated as the ratio of activity expressed by bound enzyme to the total activity used for immobilization.

Characterization of free and immobilized pectin lyase from *Bacillus cereus*

For characterizing the different parameters for free and immobilized pectin lyase, effect of different concentrations of formaldehyde (5, 10, 15 and 20% v/v), pH of glycine-NaOH buffer (8.0, 9.0, 10.0, 11.0 and 12.0), buffer molaritiy (30, 40, 50, 60 and 70 mM), reaction temperature (20, 30, 40, 50 and 60˚C), incubation time (5, 10, 15, 20, 25 and 30 min), substrates (apple pectin, pectin, polygalacturonic acid and citrus pectin), substrate concentration (0.25%, 0.50%, 0.75%, 1.0%, 1.25% and 1.5%, w/v) was studied. Thermal stability of free and immobilized enzyme was checked by incubating them at different temperatures for different time intervals. Effect of metal ions (Ca^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+} and Fe^{3+}) and recycling efficiency was also studied. Enzyme activity was determined by standard assay method**.**

Statistics

All of the tests were conducted in triplicate for determination of pectin lyase activity. A statistical analysis was done using Student's t-Test and data were expressed as means ± standard deviations.

Results and Discussion

Binding efficiency of pectin lyase onto various supports

The binding efficiency of partially purified pectin lyase from *Bacillus cereus* on agar-agar, celite-545 and chitosan was found to be 55%, 62% and 83% as shown in figure 1. Chitosan was selected further for immobilization on the basis of binding efficiency.

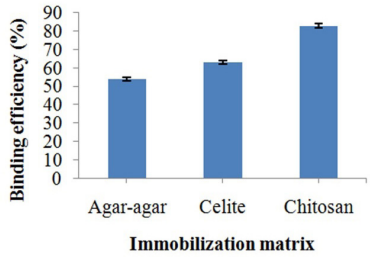

Figure 1: The binding efficiency of purified pectin lyase from *Bacillus cereus* on different matrices.

Characterization of free and immobilized pectin lyase from *Bacillus cereus*

Effect of formaldehyde concentration: In the present study, it was found that immobilized pectin lyase showed optimum activity (3.89 U/mg) at 10% concentration of formaldehyde (Figure 2). In earlier studies, the covalent binding of pectin lyase onto chitosan beads by using formaldehyde as a cross-linking agent showed higher immobilization yield as compared to entrapment of pectin lyase within calcium alginate and agar-agar [8,13].

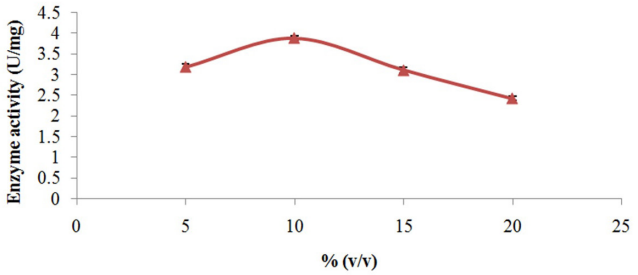

Figure 2: Effect of formaldehyde concentration on immobilization of pectin lyase from *Bacillus cereus* onto chitosan matrix.

Effect of buffer pH and molarity on free and immobilized pectin lyase: In the present study, both free and immobilized pectin lyase showed optimum activity of 3.58 U/ml and 3.89 U/mg respectively, in presence of glycine-NaOH. Buffer (50 mM) at pH 10.0 (Figure 3). This finding can be result of new environment created due to adsorption of enzyme in the matrix, which might have buffered and consequently protected the protein structure of enzyme [14]. Pectinase from *Aspergillus aculeatus* showed that the free enzyme had a pH-optimum of 5.0 while the immobilized enzyme maintained high level of activity in a broad pH range of 3.0-7.0 probably due to ionically charged surface of the PEI-functionalized polymer [15].

Pectin lyase gave maximum activity with 50 mM solution of glycine-NaOH buffer (pH 10.0) for both free (3.58 U/ml) and immobilized enzyme (3.89 U/mg) (Figure 4). In an earlier study the purified pectin lyase had shown maximum activity with 0.5M phosphate buffer [16].

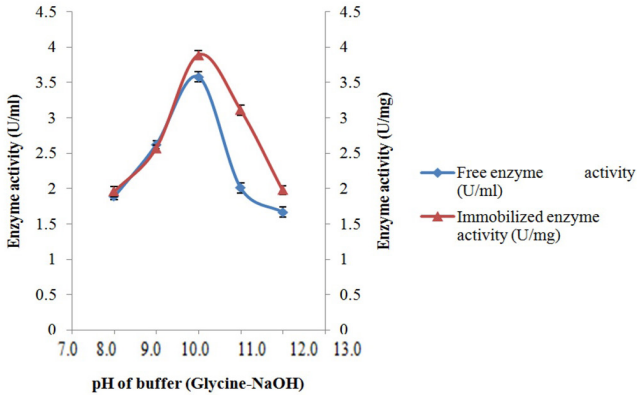

Figure 3: Effect of pH of buffer (glycine-NaOH) on the activity of free and immobilized pectin lyase.

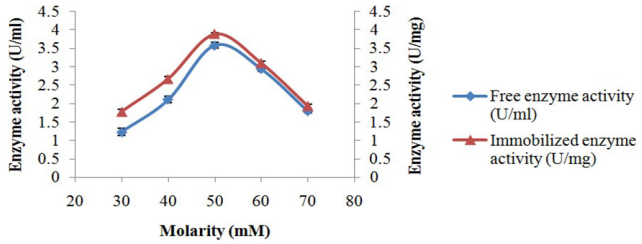

Figure 4: Effect of molarity of buffer on the activity of free and immobilized pectin lyase.

Effect of reaction temperature: In the present study the reaction temperature of 40˚C for free and 50˚C for immobilized enzyme was found to be the suitable temperature for the optimal activity (3.58 U/ml for free and 4.04 U/mg for immobilized enzyme) (Figure 5). This change can be related to enhancement of molecular diffusion rate promoted by the increase of temperature and to alterations of physical properties of enzyme after immobilization [17]. The appropriate high kinetic energy is necessary to the reorganization of the molecular structure to reach an adequate conformation to make possible the binding with the substrate, making the enzyme more resistant to heat denaturation [18]. Although diverse results on influence of temperature can be found in literature for pectin lyase, an optimal temperature of 50˚C for the soluble and 60˚C for immobilized pectin lyase from *Penicillium italicum* had been reported [19].

Effect of incubation time on free and immobilized pectin lyase: The enzyme showed maximum activity (3.58 U/ml for free and 4.04 U/mg for immobilized enzyme) after the incubation time of 15 min (Figure 6). This is comparable to a recent study, where free and immobilized commercial pectinase showed maximum activity after 20 min of incubation [20]. Further incubation beyond optimum value caused a decreased in enzyme activity, which might be due to product inhibition, when incubated for a longer period of time [21].

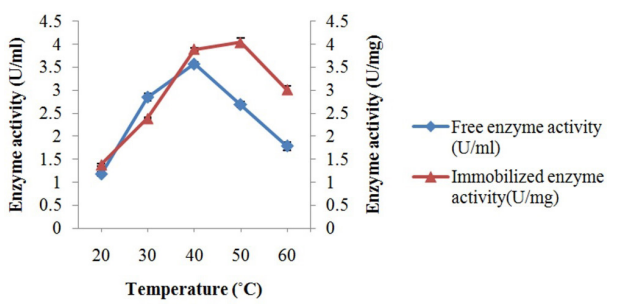

Figure 5: Effect of reaction temperature on the activity of free and immobilized pectin lyase.

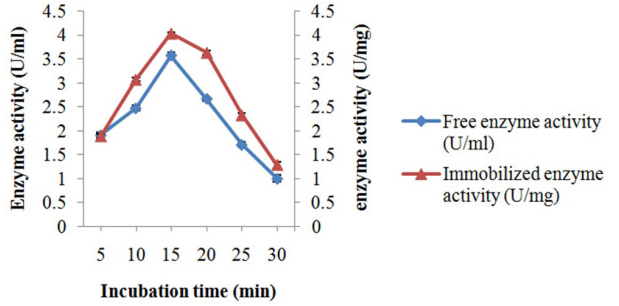

Figure 6: Effect of the incubation time on the activity of free and immobilized pectin lyase from *Bacillus cereus*.

Effect of different substrates and substrate concentration on free and immobilized pectin lyase: Pectin lyase showed optimum activity with citrus pectin as a substrate for the free and immobilized enzyme (Table1). Earlier, polygalacturonase from *Aspergillus niger* MTCC 3223 was found to have high affinity for polygalacturonic acid (0.5 %) [22]. In a previous study, results showed that pectin lyase purified with ion-exchange chromatography gave the highest activity toward citrus pectin [16].

Substrate (0.5%, w/v)	Free enzyme activity (U/ml)	Immobilized enzyme activity (U/mg)
Apple pectin	0.69±0.017	0.71±0.041
Pectin	1.41±0.032	1.80±0.028
Polygalacturonic acid	2.43±0.031	3.24±0.042
Citrus pectin	3.58±0.027	4.04±0.021

Table 1: Effect of different substrates on the activity of pectin lyase from *Bacillus cereus*.

Pectin lyase gave optimum activity with citrus pectin as substrate at a concentration of 0.5% (w/v) for free as well as immobilized enzyme (Table 2). It was elucidated from an earlier investigation that pectin lyase production from *Streptomyces* sp. was stimulated by pectin and the optimized substrate concentration gave the highest pectin lyase activity of 1520 U/ml from 0.3% w/v pectin [23].

Thermostability of free and immobilized pectin lyase: Thermal stability of free and immobilized pectin lyase was investigated.

Half-life of free enzyme at 40, 50 and 60˚C was 120, 90 and 60 min respectively (Figure 7a) whereas, 150, 120 and 90 min for immobilized enzyme at 40, 50 and 60˚C respectively (Figure 7b). The increased stability of immobilized enzyme could be ascribed to the enhancement of enzyme rigidity and conformational flexibility by immobilization, preventing the conformational change at high temperatures [24]. It was reported earlier that the half-life times of free and immobilized pectinase protected 60 and 70% of their initial activities respectively at 35˚C [15].

Substrate concentration (%, w/v)	Free enzyme activity (U/ml)	Immobilized enzyme activity (U/mg)
0.25	1.58±0.025	2.58±0.015
0.5	3.58±0.028	4.04±0.023
0.75	2.95±0.032	2.99±0.028
1	1.89±0.019	1.95±0.031
1.25	1.41±0.026	1.19±0.039
1.5	0.82±0.017	0.87±0.026

Table 2: Effect of substrate concentration on the activity of pectin lyase from *Bacillus cereus*.

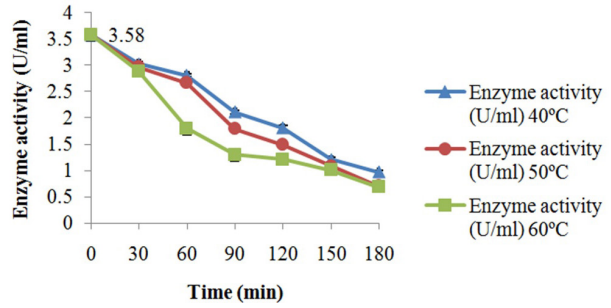

Figure 7a: Thermostability profile (at 40, 50 and 60˚C) of free pectin lyase from *Bacillus cereus*.

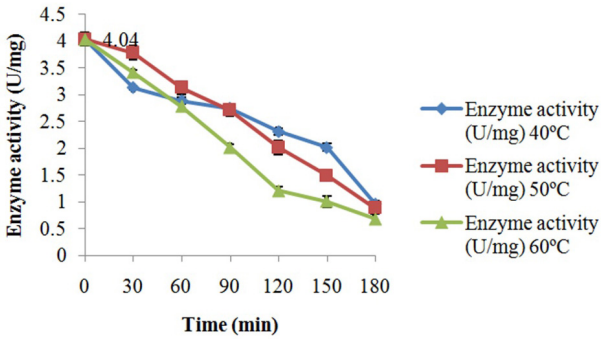

Figure 7b: Thermostability profile (at 40, 50 and 60˚C) of immobilized pectin lyase from *Bacillus cereus*.

Effect of metal ions: Pectin lyase from *Bacillus cereus* showed maximum enzyme activity in the presence of Mg^{2+} ions for both free (3.59 U/ml) and immobilized enzyme (4.06 U/mg). However, Cu^{2+} and Zn^{2+} also enhanced the activity of free and bound pectin lyase (Table 3). In a study, the Fe^{3+} ascorbic acid and Ca^{2+} strongly activated pectin lyase, and Na^{+}, Zn^{2+} and Mg^{2+} partially activated pectin lyase, K^{+} partially inhibited pectin lyase and Mn^{2+} had no effect on pectin lyase activity [16].

Metal ion (1mM)	Free enzyme activity (U/ml)	Immobilized enzyme activity (U/mg)
Ca^{2+}	1.79±0.02	1.98±0.03
Cu^{2+}	2.45±0.05	3.11±0.07
Mg^{2+}	3.59±0.02	4.06±0.02
Zn^{2+}	1.94±0.07	3.14±0.06
Mn^{2+}	1.41±0.03	1.91±0.04
Fe^{3+}	1.06±0.05	1.39±0.05
Control	0.95±0.07	1.01±0.02

Table 3: Effect of metal ions on the activity of pectin lyase from *Bacillus cereus*.

Reusability of immobilized pectin lyase: Immobilized pectin lyase retained almost 50% of its original activity up to 4th cycle (Table 4). In earlier study, polyglacturonase from *Aspergillus niger* immobilized on nylon-6 retained about 50% of its activity up to four cycles [25]. The pectinases immobilized onto modified nano-silica and florosil retained 80 and 76% of their initial activities respectively up to 10 cycles [15].

Cycle	Immobilized enzyme activity (U/mg)
1	4.04±0.03
2	3.76±0.05
3	2.91±0.01
4	2.05±0.05
5	1.56±0.04
6	1.01±0.07
7	0.78±0.02

Table 4: Reusability of the immobilized pectin lyase from *Bacillus cereus*.

Conclusion

The covalent binding of pectin lyase on chitosan beads was found to be promising technique for the immobilization of pectin lyase. The thermostability of pectin lyase was increased after immobilization as compared to free enzyme. It exhibited reusability and retained more than 50% activity even after reusing it in the reaction 4 times. The improvement of temperature stability as well as reusability of pectin lyase after immobilization enhanced the potential of pectin lyase to be applicable in various industrial preparations.

Acknowledgement

The Financial support from Department of Biotechnology, Ministry of Science and Technology, Government of India, to Department of Biotechnology, Himachal Pradesh University, Shimla (India), is thankfully acknowledged. The financial assistance to Ms. Pooja Kohli as RGNF from UGC and to Kamal Kumar Bhardwaj as CSIR fellow is thankfully acknowledged.

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